Novel Methods for the Detection of Mephedrone and Anabolic Steroids in Hair using Liquid Chromatogrphy Tandem Mass Spectrometry

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V Novel Psychoactive Substance Conference,
Vienna 23-24/10/17

Parallel Session 5: New approaches to detecting and characterising NPS

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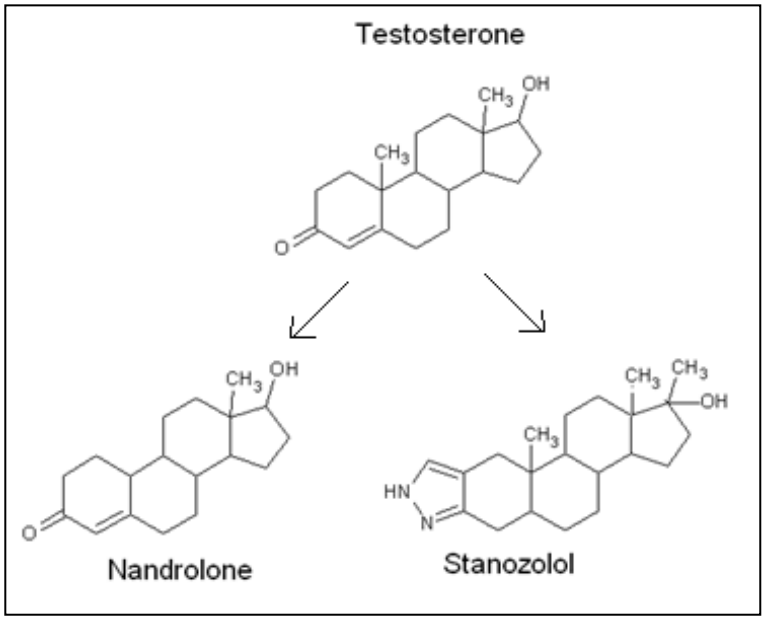
Prohibited Substances

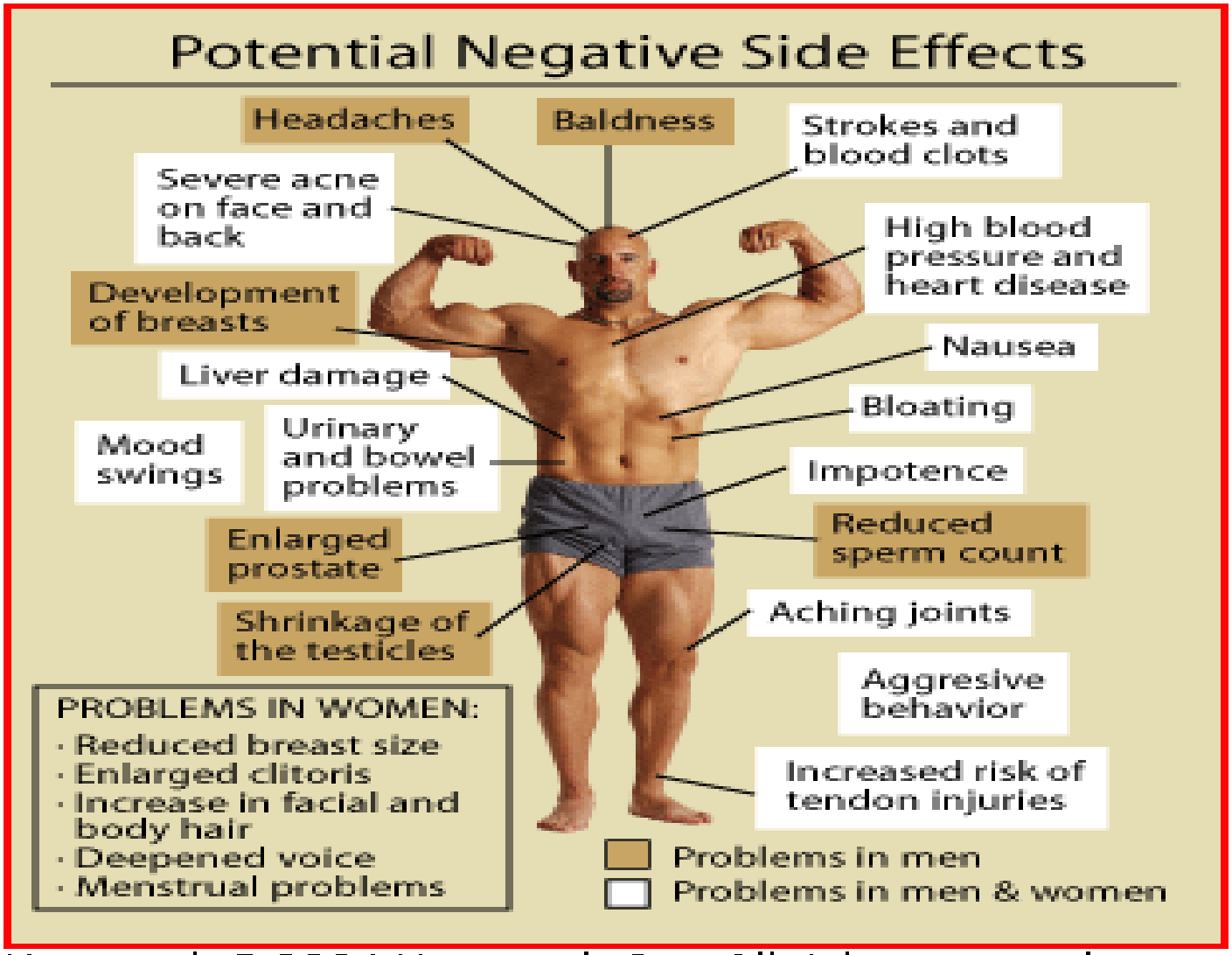
1. Anabolic Agents e.g.; Testosterone, Nandrolone *etc*
2. Hormones e.g.; Erythropoetin, Insulin *etc*
3. Diuretics e.g.; Albumin, Spironolactone *etc*
4. Beta-2-Agonists e.g.; Clenbuterol, Salbutamol, *etc*

Anabolic, Androgenic Steroids (AAS)

- Anabolic effect
  - Accelerate muscle growth (protein synthesizing)
- Androgenic effect
  - Develop masculine characteristics
- Anabolic androgenic steroids (AASs) are of two types:
  - Endogenous
    - Produced naturally in the body
      e.g. Testosterone (male sex hormone)
  - Exogenous
    - Synthetic derivatives of testosterone
      e.g. Nandrolone, Stanozolol



Dangers of Anabolic Steroids



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Challenges faced by sports federations

- Urinalysis susceptible to false doping results
  - e.g. Inter-individual and inter-ethnic variations in the prevalence of deletion polymorphism in gene coding of *UGT2B17 (a steroid metabolizing enzyme)* affects amount of glucuronidated metabolites produced
- Urine provides a short term history of drug use
  - Disadvantage in detecting out of competition doping
- Hair analysis, a possible approach to complement urinalysis

AAS

- Potentially misused as training drug for performance enhancement
- Use prohibited in and out of competition – World Anti-Doping Agency (WADA)
- Generally, doping with exogenous and endogenous AAS is determined by:
  - Detecting glucuronide conjugates of the parent AAS and/or their Phase I metabolites in urine
- Supraphysiological administration of Testosterone is suspected when:
  - The urinary ratio of Testosterone glucuronide (TG) to Epitestosterone glucuronide (EG) exceeds 4 (TG/EG>4)
- Epitestosterone (E) is an inactive epimer of Testosterone (T)
- *UGT2B17*
  - Major enzyme involved in glucuronidation of most AASs [1]

1**. Deshmukh N**, Petróczi A, Barker J, Székely AD, Hussain I, Naughton DP. Potentially harmful advantage to athletes: a putative connection between UGT2B17 gene deletion polymorphism and renal disorders with prolonged use of anabolic androgenic steroids. *Substance Abuse Treatment, Prevention, and Policy* 2010, **5**:7

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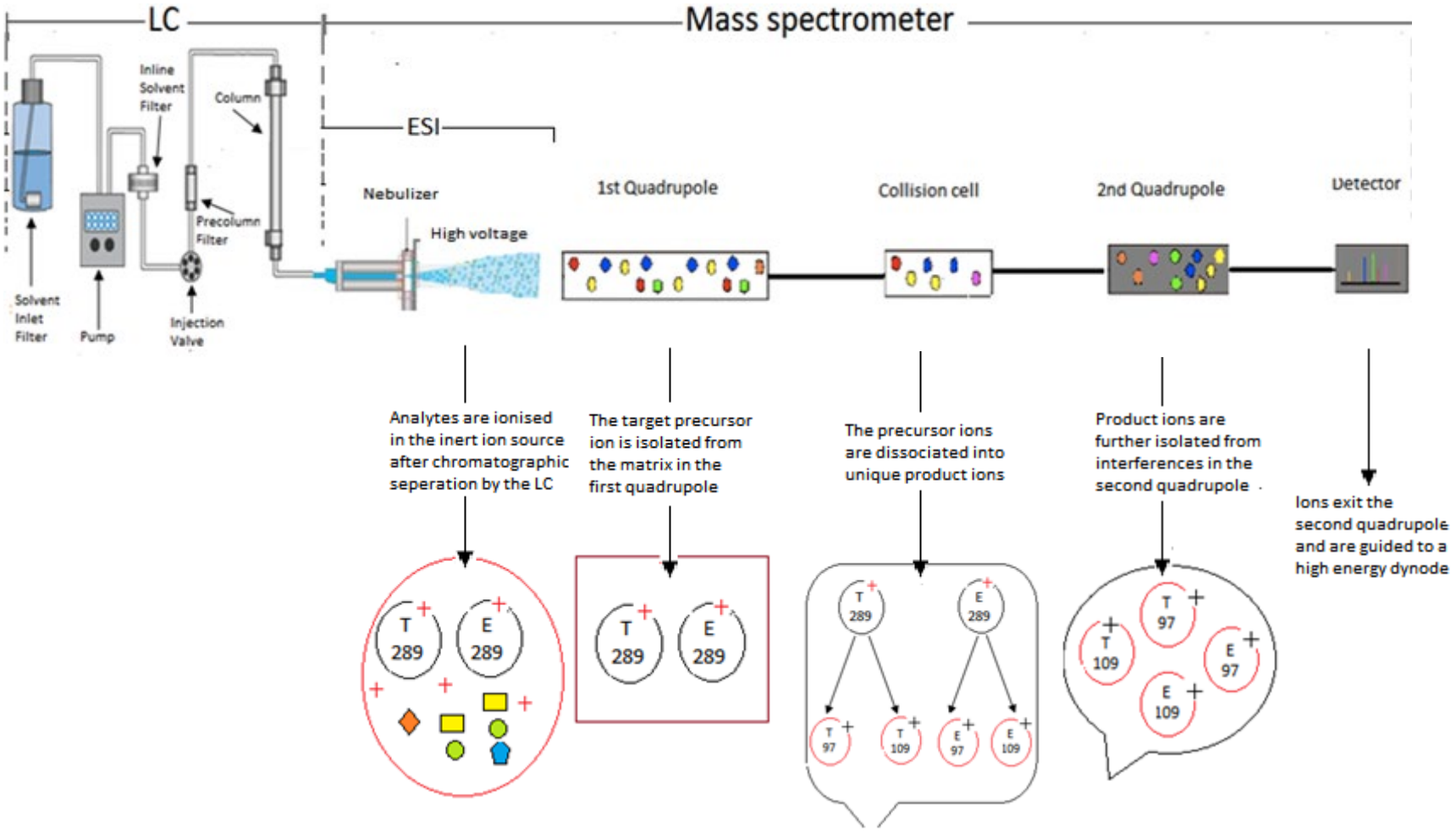
Advantages of hair specimens over urine

- Provides long term history of drug use, hence can identify athletes who use AAS during training and stop their use before competition
- Capable of facile detection of parent drug
- Easy to collect and store
- Tamper resistant and non-intrusive
- Negligible risk of infection

AIMS

- To develop new methods for the determination of nandrolone and stanozolol in human hair using LC-MS/MS
- To extend these newly developed methods for the determination of testosterone (T) and its inactive epimer epitestosterone (E) in human hair
- Thus, to estimate the physiological ratio of T/E in hair
- To study effect of pigment and use of other drugs on incorporation of AAS in hair
- To measure environmental contamination into potable water supplies of steroids

Liquid chromatography tandem mass spectrometry



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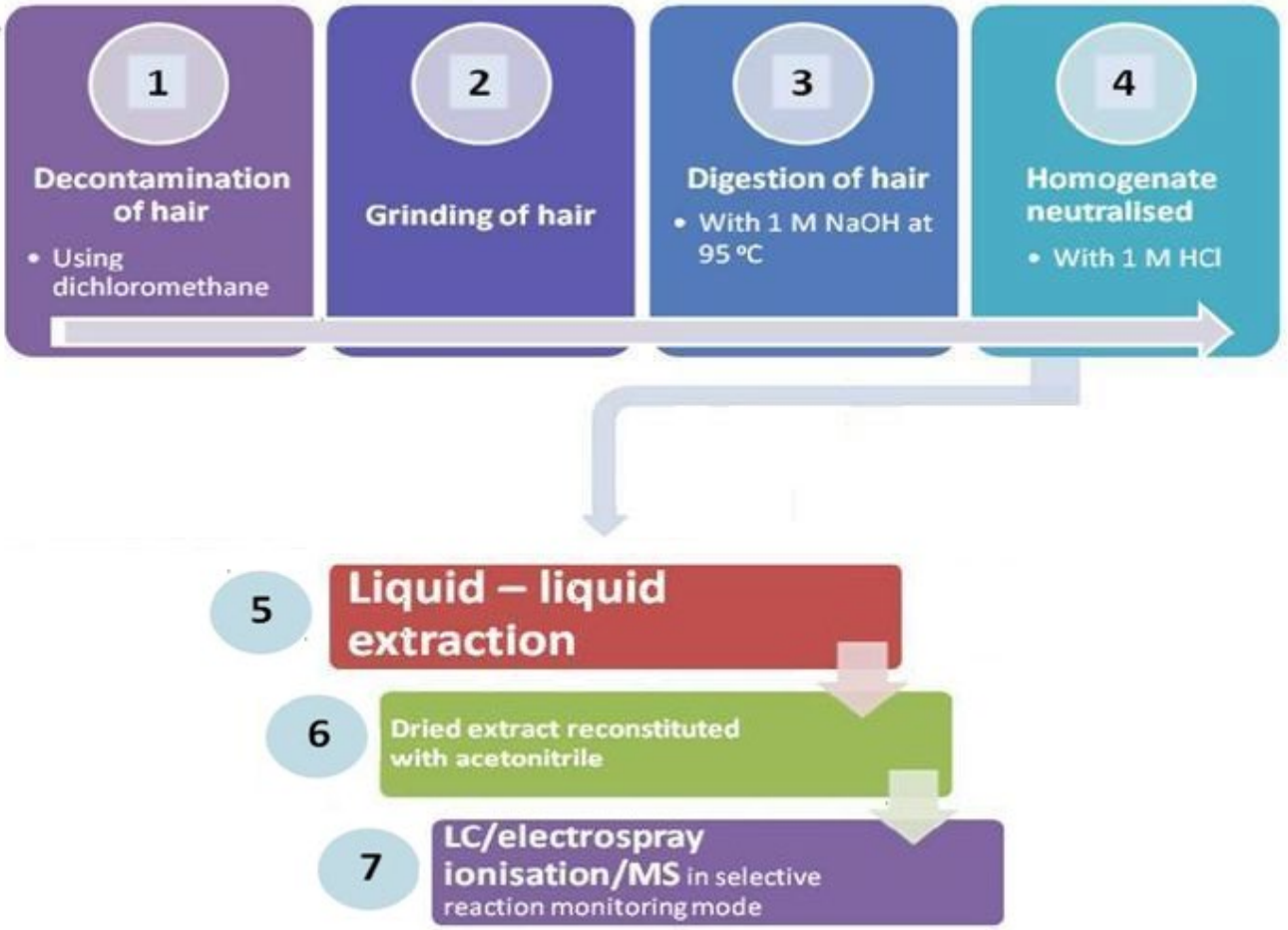
SAMPLE PREPARATION



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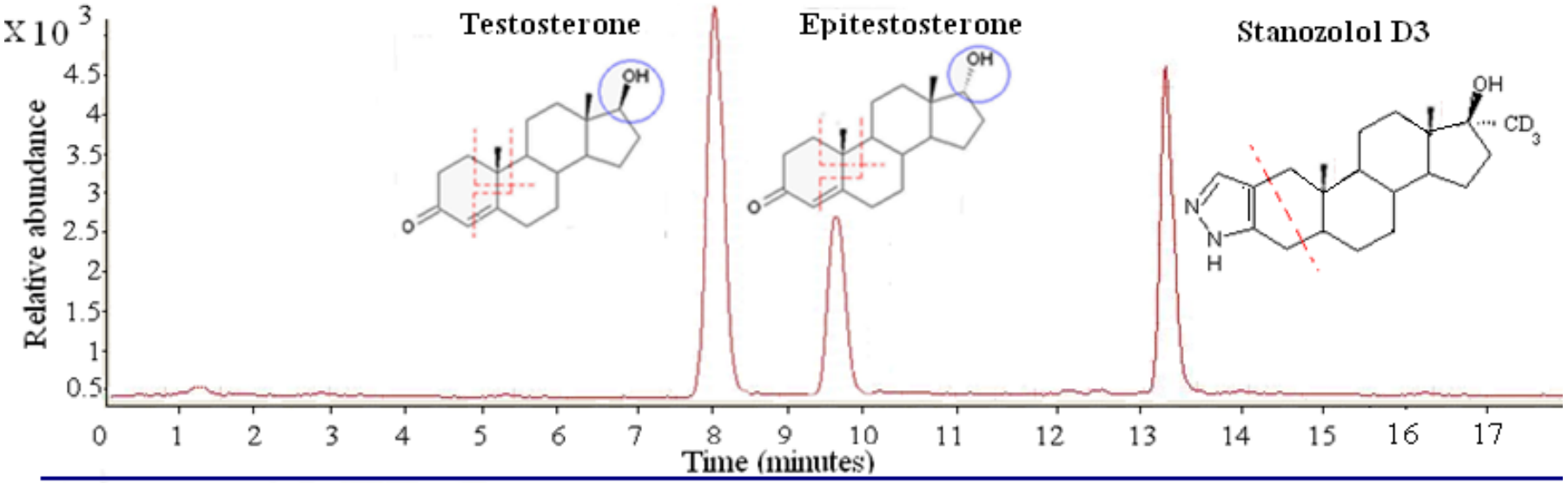

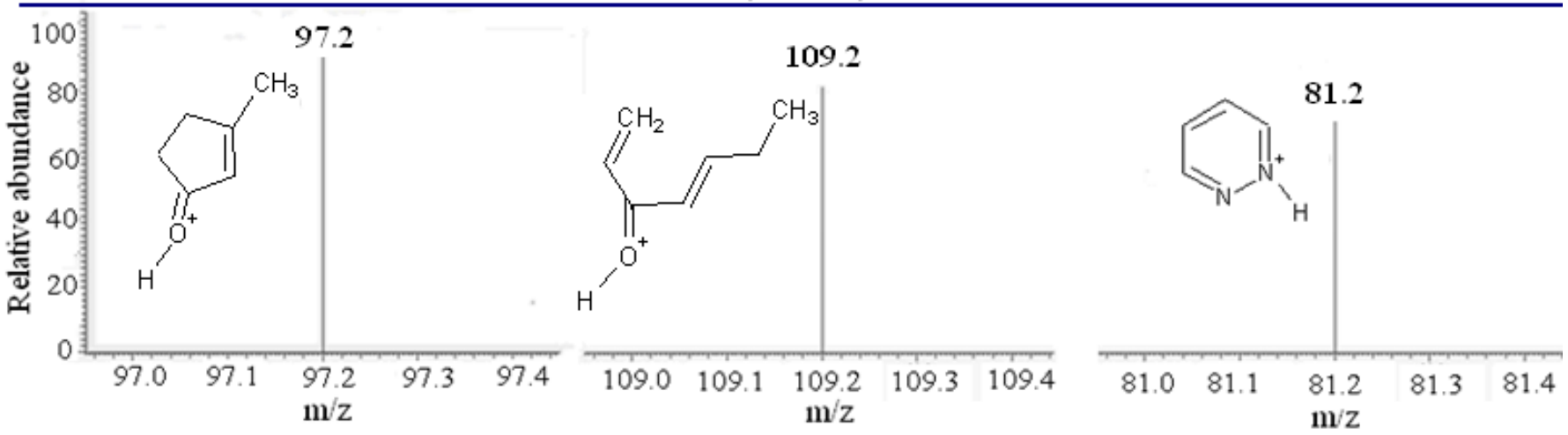

Product ions of Testosterone and Epitestosterone

Product ion of Stanozolol D3

APPLICATIONS

Concentration of nandrolone and stanozolol in human hair (n=180, 108 Male, 72 female, 62% athletes) ELISA: 16 +ve Stanozolol, 3 +ve Nandrolone

	Sex	Age	Stanozolol pg/mg	Nandrolone pg/mg
Control *	M	53	47.4	-
Athlete 1	M	22	5.0	-
Athlete 2	M	19	11.2	-
Athlete 3	M	21	33.0	-
Athlete 4	F	18	-	14.0
Athlete 5	F	19	9.8	-
Athlete 6	F	22	10.0	-
Athlete 7	F	20	12.7	-
Athlete 8	F	20	26.9	-
Athlete 9	F	18	40.2	-
Athlete 10	F	20	56.1	-
Athlete 11	F	22	63.3	-
Athlete 12	F	20	86.3	-

*Male bodybuilder who admitted use of stanozolol for the past 30 years by intramuscular and oral route

Determination of stanozolol in environmental and domestic water samples

Environmental water sample (N=3)	Average concentration pg/mL					
	31st December 2009	18th April 2010	21th July 2010	01st September 2010	24th October 2010	05th November 2010
River Danube	1.82 ± 0.19	0.71 ± 0.06	0.54 ± 0.03	ND	ND	ND
Budapest Tap	1.19 ± 0.03	0.31 (BLQ)	ND	ND	ND	ND
Lake Balaton	-	ND	-	-	-	-
Spring 'Rózsika'	-	ND	-	-	-	-

Reasons for Reduction

- Variations in rates of contamination. In July 2010 a new STP was commissioned.
- Dilution of river water due to rise in water levels.
- Degradation of the steroid in the river water due to other constituents in the river or photolysis *etc.* or deposition in the sediment.
- 3'Hydroxystanozolol, the metabolite was not detected.

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Concentration of testosterone and epitestosterone in human hair

Subject	Male (n=49)		
Age range	18 to 42 years		
Analyte	Testosterone (T)	Epitestosterone (E)	T/E ratio
Range	0.70 to 11.81 pg/mg	0.63 to 8.27 pg/mg	0.50 to 3.37
Average	2.67 pg/mg	2.46 pg/mg	1.33
Median	1.98 pg/mg	1.86 pg/mg	1.11
Standard error	0.29	0.34	0.13

Subject	Female (n=26)		
Age range	18 to 55 years		
Analyte	Testosterone	Epitestosterone (detected in only 7)	T/E ratio
Range	0.32 to 4.63 pg/mg	0.52 to 3.88 pg/mg	0.56 to 1.81
Average	1.62 pg/mg	1.73 pg/mg	1.2
Median	1.03 pg/mg	1.23 pg/mg	1.30
Standard error	0.27	0.45	0.17

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Concentration of natural testosterone in pigmented and non-pigmented hair of rats

Animal	Concentration of testosterone (pg/mg)		Ratio of pigmented to non-pigmented
	Pigmented hair	Non-pigmented hair	
1	6.33	4.8	1.32
2	8.74	1.81	4.83
3	6.14	1.51	4.07
4	7.84	1.84	4.26
5	5.88	1.58	3.72
6	4.84	1.28	3.78
7	5.61	1.49	3.77
8	3.66	1.11	3.30
9	3.06	1.41	2.17
10	4.81	1.14	4.22
11	2.86	0.84	3.40
Mean ± SD	5.43 ± 1.85	1.71 ± 1.07	3.53 ± 1.00

Influence of NSAIDs on Stanozolol excretion

Group	Individual animals	Diclofenac
G1	No. 1-6	0 mg/kg
G2	No. 7-12	1 mg/kg
G3	No. 13-18	5 mg/kg
G4	No. 19-24	25 mg/kg

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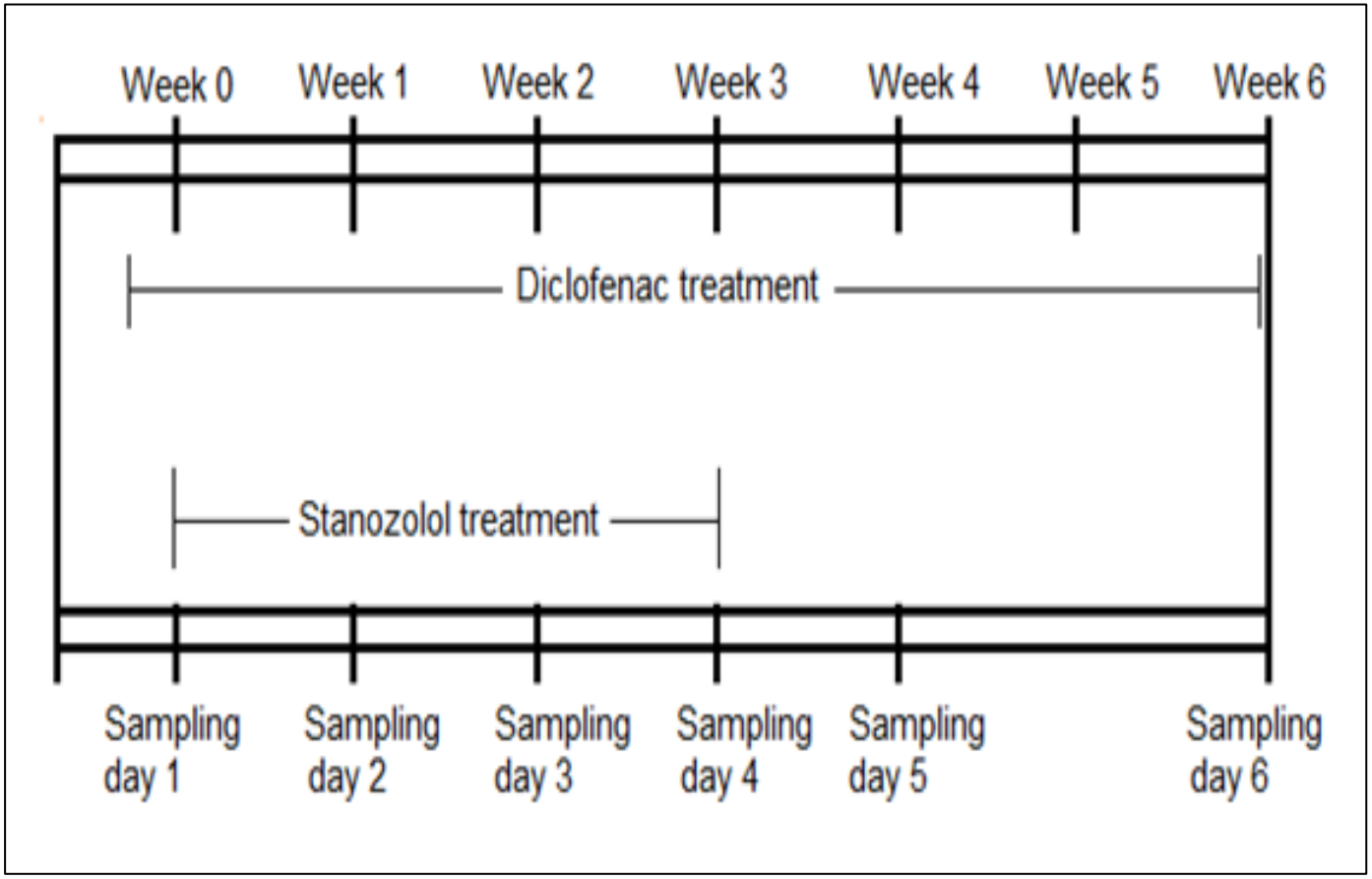
Drug treatment and sample collection period



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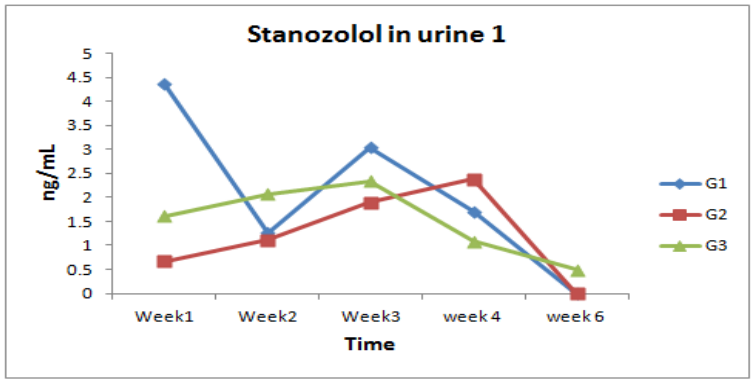
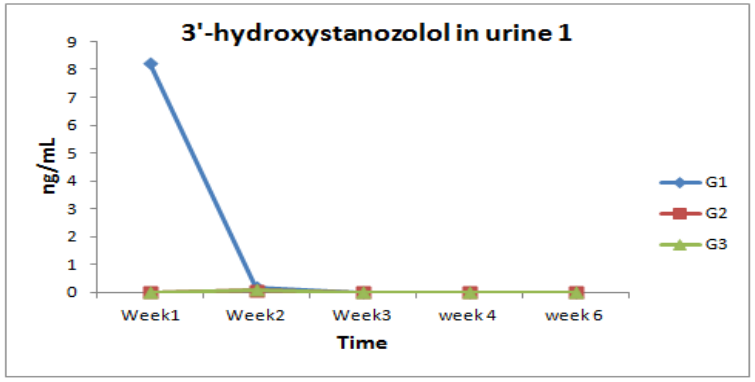
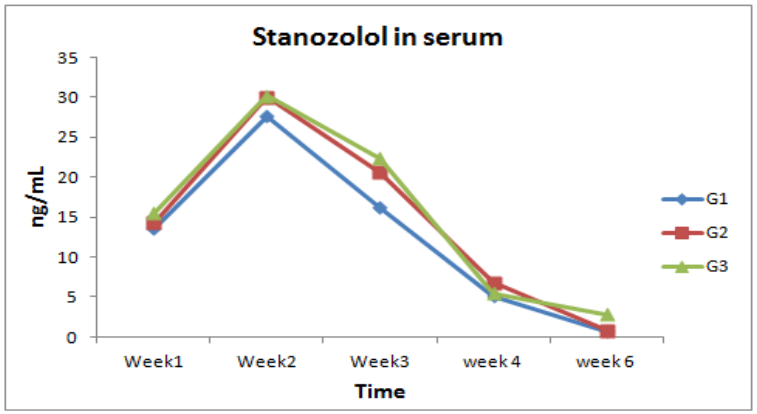
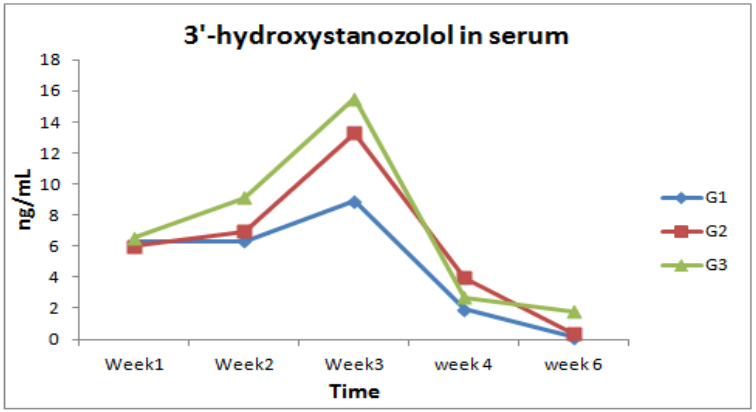
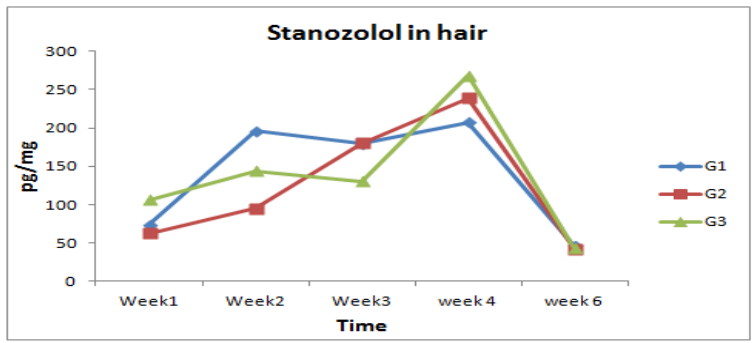
Concentrations of stanozolol and 3'-hydroxystanozolol in hair samples from groups G1, G2 and G3 during stanozolol treatment (week 1, week 2 and week 3) and poststanozolol treatment period (week 4 and week 6)

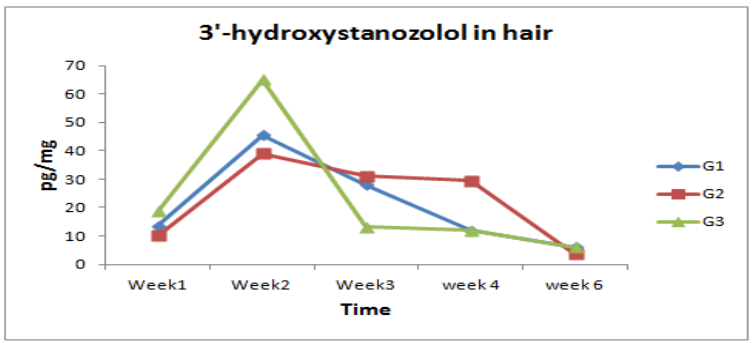












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CONCLUSIONS

- Methods developed are sensitive, specific, reliable and reproducible.
- LC-MS analysis does not require derivatisation unlike GC-MS.
- 20-50 mg hair required to detect 3 pg/mg nandrolone, 0.5 pg/mg stanozolol, 0.1 pg/mg testosterone and 0.25 pg/mg epitestosterone.
- Hair testing facilitates improved doping testing regimes that are non invasive, tamper resistant and benefit from facile storage and negligible risk of infection.
- Hair specimens can be used to compliment urinalysis.
- Concomitant use of other drugs (NSAIDs) and hair pigmentation affect levels of steroids.

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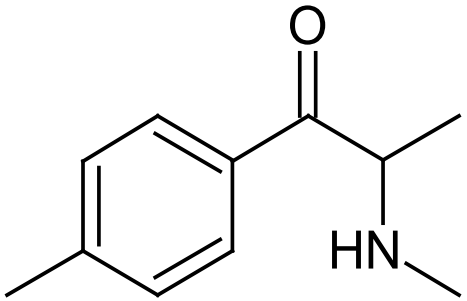
Mephedrone



- Former “legal high” which shows psychoactivity in humans
- Sometimes referred to as ‘meow’, ‘mcat’ and ‘bubbles’
- It is a β-ketoamphetamine and has a structure similar to methcathinone
- Routinely sold on the internet as plant feed
- In April 2010, it became a controlled drug under the Misuse of Drugs Act 1971 as a Class B substance (UK)

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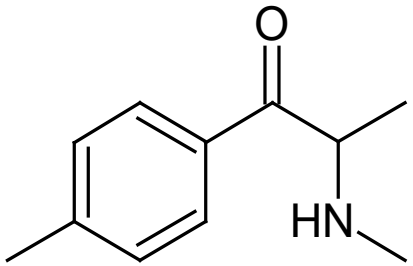
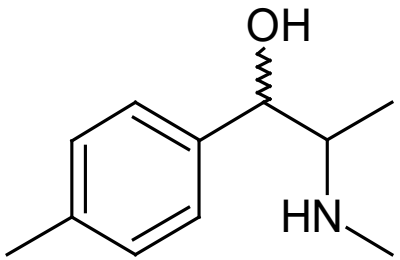
Aim:

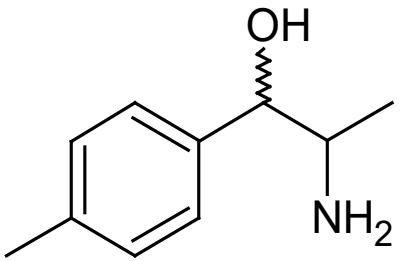
The aim of the study was to develop a validated sensitive and reproducible method for the quantification of mephedrone and its two metabolites 4 – methylephedrine and 4 – normethylephedrine in human hair using liquid chromatography – mass spectroscopy.



mephedrone



4 – methylephedrine



4 – methylnorephedrine

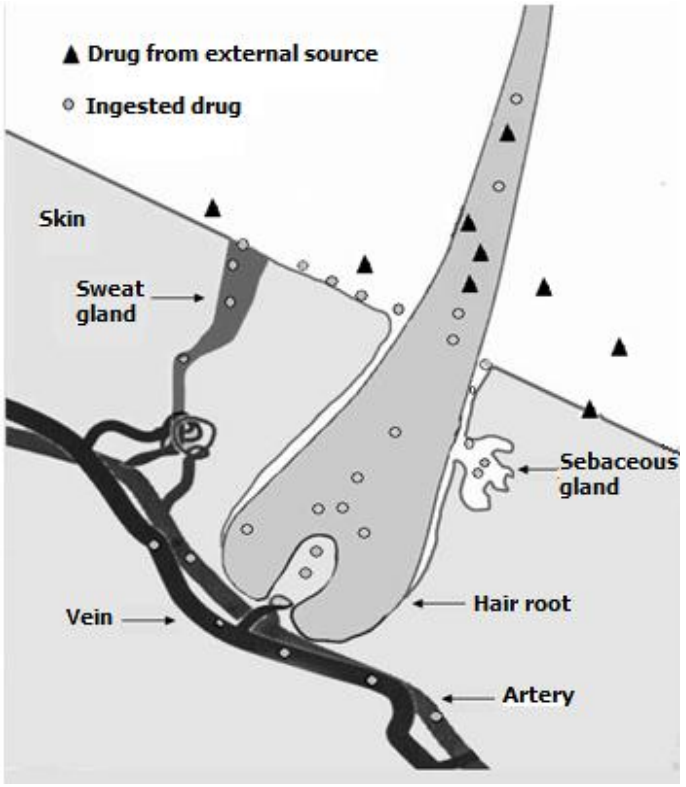
Incorporation of drugs in hair

Three pathways

1. **Endogenous pathway:** Passive diffusion of drugs from systemic circulation into hair via follicles

2. **Endogenous–exogenous pathway:** Absorption or transfer of drug molecules into hair shaft from transdermal excretion, sweat and sebum

3. **Exogenous pathway:** Drugs deposited from external environment enter hair by absorption



• Drug incorporation usually happens during the synthesis of melanin, where it gets bound to the melanin polymer.

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Why use hair?

- It provides information on the long term history of the individual's intake of drugs
- Any kind of hair treatment such as bleaching cannot affect the incorporation of the drug into hair
- It is a non-invasive technique
- Easy storage, minimal degradation, Biosafe
- It can be used to compliment urinalysis for identifying routine drug users

Compound	(RT) min	Precursor ions (m/z)	Product ions(m/z)	Collision energy (eV)
Mephedrone	2.07	178.1	160.1	10
			145.1	18
4-methylephedrine	1.93	180.2	147.2	19
			131.3	18
			115.2	39
4-methylnorephedrine	1.85	166.2	131.2	14
			115.2	33
			90.79	24
Mephedrone –d3	1.98	181.2	163.2	17
			148.2	20

Ion transitions, retention time (RT) and collision energies used for the analysis of the analytes and the internal standard.

- Capillary temp: 300^0C
- Voltage: 4000V
- Collision pressure: 1.5mTorr

Sample preparation:

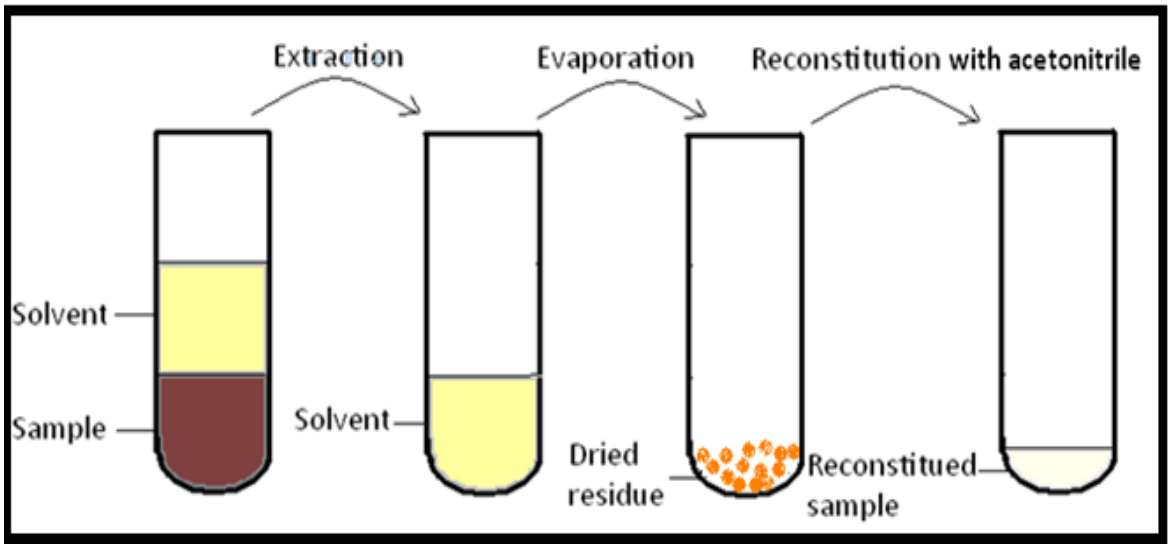
Hair (50 mg) decontaminated (DCM) and pulverised

↓

Enzymatic hair digestion with Proteinase K and Cleland's Reagent

↓

Liquid-liquid extraction



Method Validation

Compound	Concentration (pg/mg)	Precision RSD (%)		Accuracy %
		Intraday N=6+6+6 +6	Interday N=18+18+18+18	
Mephedrone	5	0.62	0.8	99.15
	10	0.4	0.7	106.34
	20	2.11	1.7	103.16
	40	2.56	4.5	107.53
4 – methylephedrine	10	0.6	0.9	114.59
	20	0.75	0.7	110.76
	50	0.8	4	107.02
	100	4.92	3.4	98.52
4–methylnorephedrine	10	0.54	1.1	107.98
	20	0.2	0.7	106.51
	50	2.91	2	97.6
	100	6.97	7.3	100.67

Compound	Recovery (%) at Conc. (20 pg/mg)	LOD (pg/mg)	LOQ (pg/mg)
Mephedrone	110.37	2.5	5
4-methylephedrine	98.37	5	10
4-methylnorephedrine	96.78	5	10

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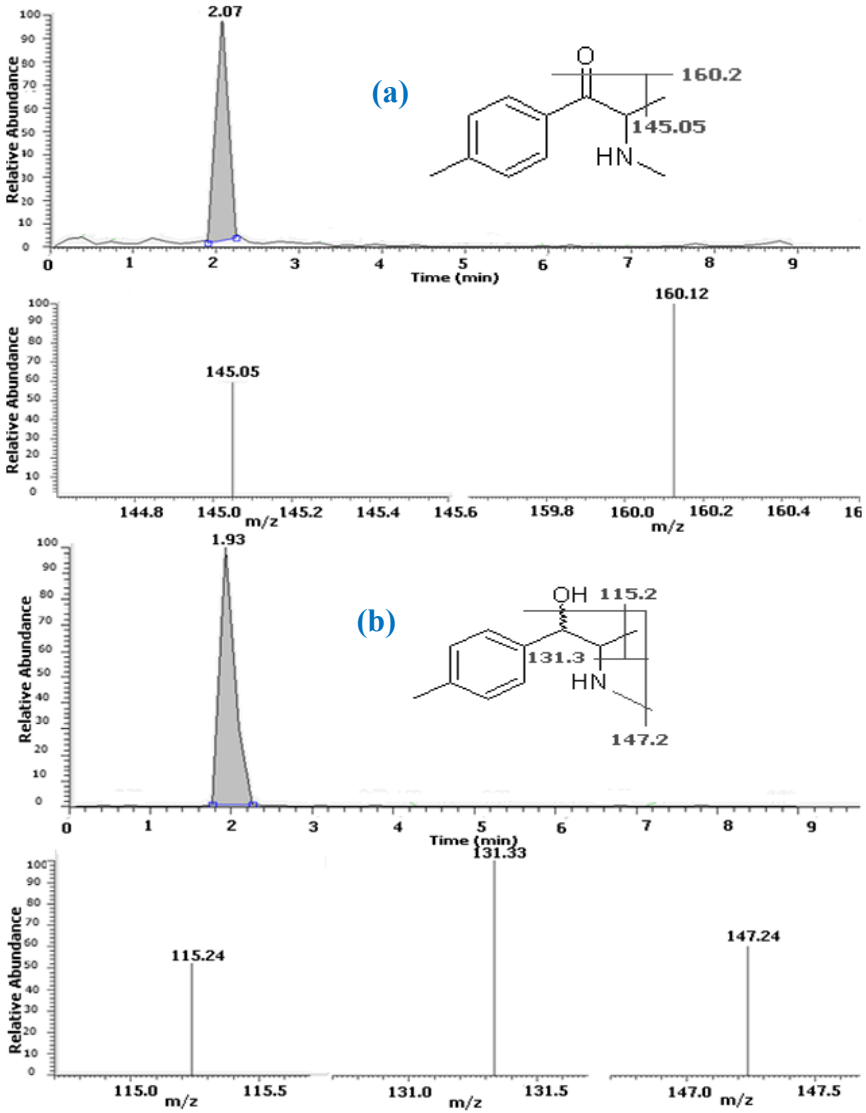
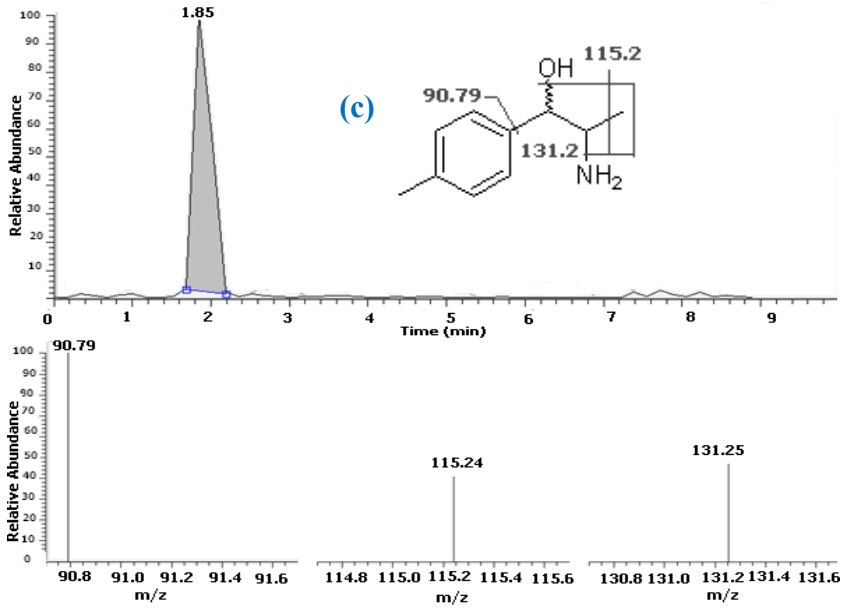
LC–MS/MS chromatogram and CID spectra of (a) mephedrone, (b) 4-methylephedrine and (c) 4-methylnorephedrine in hair at concentrations of 10pg/mg, 20pg/mg and 20pg/mg respectively.





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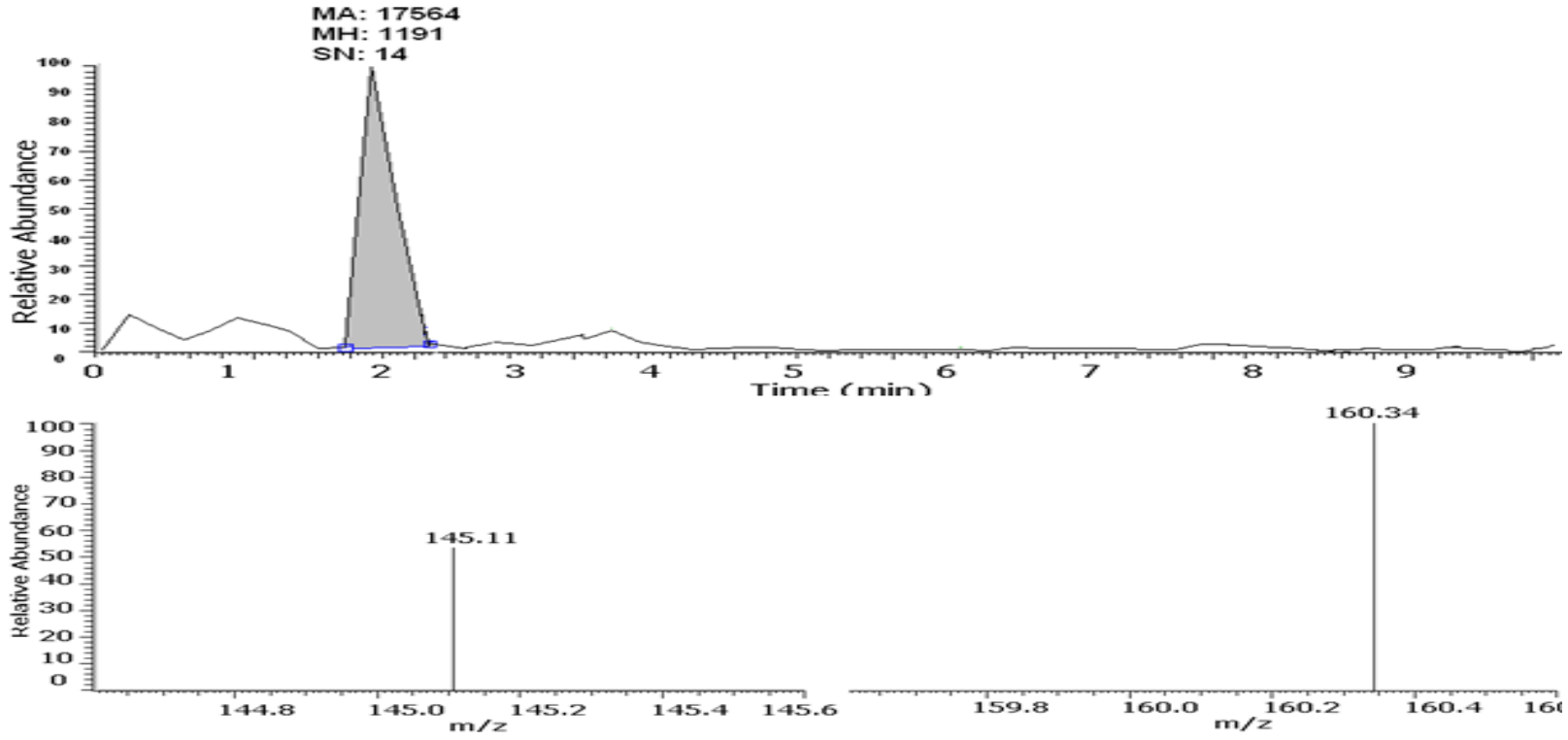
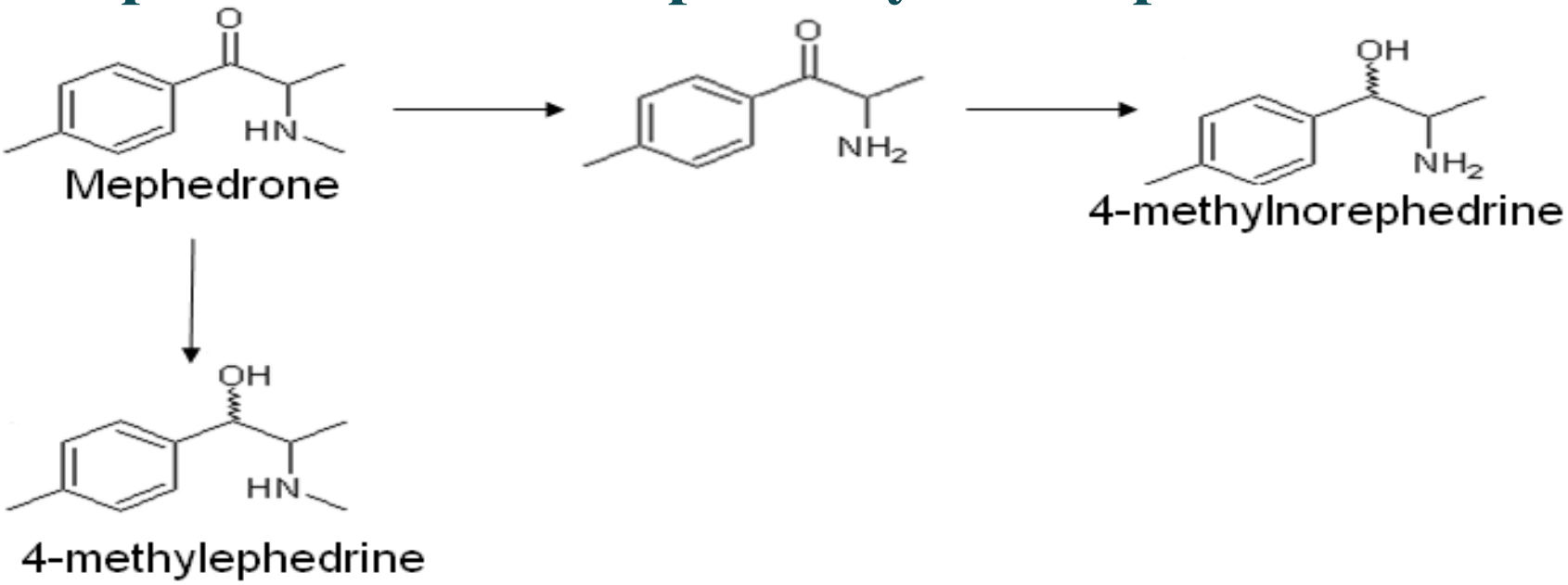
• Out of 154 samples, mephedrone could be detected in four samples and quantified in only one hair sample at a concentration of 21.11 pg/mg.



LC–MS/MS chromatogram and CID spectra of the positive sample

Proposed metabolism pathway for mephedrone



- Due to the structural similarities of mephedrone to methcathinones and methamphetamines, the metabolism of mephedrone has been proposed to follow a similar pathway.
- There is a possibility of other pathways
- The metabolites were not detected in any of the samples analysed.

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PUBLICATIONS

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THANK YOU FOR YOUR ATTENTION

No substitute for hard work…!